Molecular characterisation and hormone-dependent expression of the porcine whey acidic protein gene

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ABSTRACT

A 17·5 kDa protein was isolated from porcine whey by reverse phase HPLC and identified as a putative whey acidic protein (WAP) homologue by sequencing 35 and 40 amino acid residues of the amino- and carboxy-terminus respectively. Degenerate oligonucleotides to both of these amino acid sequences were designed and used in reverse transcriptase PCR with RNA from lactating porcine mammary gland as a template. A 162 bp PCR fragment was detected and sequenced. Compilation of the deduced and determined amino acid sequence revealed a protein of 111 amino acids, which had approximately 75, 50, 40 and 35% similarity at amino acid level to camel, rabbit, rat and mouse WAP respectively. It also included the four-disulphide core characteristic of all WAP proteins and most Kunitz-type protease inhibitors. This provides the first unequivocal evidence for WAP secretion in the pig. SDS PAGE analysis of the whey fraction showed that WAP is secreted as a major protein in sow's milk from farrowing to weaning. The molecular mass of WAP in SDS PAGE was significantly greater than the 11·7 kDa determined from amino acid sequence, indicating that porcine WAP is possibly glycosylated.

Northern analysis detected a single mRNA transcript of approximately 600 bp in porcine RNA from the mammary gland of lactating sows. To examine the hormone-regulated expression of the WAP gene the mammary glands of sows at day 90 of pregnancy were biopsied and explants cultured for 3 days in the presence of various combinations of porcine insulin (I), cortisol (F) and porcine prolactin (P). Northern analysis of RNA extracted from the tissue indicated that WAP gene expression was barely detectable in the mammary gland prior to culture and there was no increment in explants cultured in the presence of I and F. However, a significant increase in the accumulation of WAP mRNA was observed in explants cultured in I, F and P. A similar result was observed for β-casein and α-lactalbumin gene expression.
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INTRODUCTION

Major milk protein genes have been cloned from many laboratory and livestock species and the organisation and hormone-regulated expression of these genes have been studied (Groenen & van der Poel 1994). In the pig, six major milk protein cDNAs have been cloned and sequenced, including the four caseins, αs1- (Alexander & Beattie 1992a), αs2- (Alexander et al. 1992), β- (Alexander & Beattie 1992b) and κ-casein (Levine et al. 1992), and two whey proteins, β-lactoglobulin (Alexander & Beattie 1992c) and α-lactalbumin (Das Gupta et al. 1992). Whey acidic protein (WAP) is the major whey protein in the milk of the mouse (Hennighausen et al. 1982), rat (Campbell et al. 1984), rabbit (Devinoy et al. 1988) and camel (Beg et al. 1986) but has not previously been identified in the milk of any livestock species, including pigs. Indeed, the transgenic pig has been used as a model system to examine the expression of the mouse WAP gene (Shamay et al. 1991, Wall et al. 1991)

EXHIBIT

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and constructs that were prepared with the mouse WAP gene promoter (Velander *et al.* 1992).

WAP can prove difficult to identify in new species by SDS polyacrylamide gel electrophoresis because it demonstrates a range of mobilities with apparent sizes varying from 14 to 28 kDa both within and across species (Hennighausen *et al.* 1982, Krozowski 1989, Grabowski *et al.* 1991, Nicholas *et al* 1995). This range of apparent molecular weights is most likely a reflection of the degree of glycosylation of the protein. In addition, while it is likely that WAP from different species share the same tertiary structure folding pattern, a comparison of amino acid sequence reveals limited similarity. However, WAP and the Kunitz family of protease inhibitors, are recognised by the highly conserved cysteine residues which fall into a two-domain pattern, known as the four-disulphide core (Hennighausen & Sippel 1982).

We report here, for the first time, the isolation and purification of WAP from a livestock species. The identity of porcine WAP was confirmed by amino- and carboxy-terminal sequence and PCR analysis of the remaining coding region of the cDNA. Data base searches revealed similarity at the amino acid level to WAP from other species and to proteins from the four-disulphide core family. Porcine WAP is shown to be regulated in a hormone-dependent fashion, requiring cortisol, insulin and prolactin for expression in cultured mammary gland explants from pregnant sows.

MATERIALS AND METHODS

Porcine insulin (lot number 154–YB–9) was a gift from Eli Lilly Co, Indianapolis, IN, USA. Porcine prolactin (lot number AFP–5000) was generously supplied by the USDA Animal Hormone Program, Beltsville, USA. All tissue culture reagents were purchased from Gibco BRL (Grand Island, NY, USA). Sows were obtained from the Werribee Research Piggery, Victorian Institute of Animal Science, Werribee, Australia.

Isolation of porcine WAP

Milk samples were collected (Attwood & Hartmann 1992) from sows at mid-lactation. The skim milk was prepared by centrifugation of milk at 3000 *g* for 15 min and removal of the fat layer. The whey fraction was separated from casein micelles by centrifugation at 50 000 *g* for 30 min at 12 °C. WAP was purified from whey by HPLC using a POROS R2/H reverse phase column (4·6 × 100 mm) and a

15–60% gradient of acetonitrile in 0·1% trifluoroacetic acid. The absorbance of the protein peaks was measured at 215 nm. The protein fractions were dried under vacuum and stored at 4 °C.

Amino acid sequence determination

The amino-terminal amino acid sequence of porcine WAP was determined to 35 residues on an Applied Biosystems (ABI) 477A pulsed–liquid–phase sequencer with an on-line 120A PTH analyser. The same instrument was used for sequence analysis of peptide fragments produced by enzymic and acidic cleavage at Asp–Pro sequences (Shaw *et al.* 1993) of porcine WAP resulting in the resolution of 40 carboxy-terminal amino acids.

PCR and cloning of porcine WAP

Oligonucleotides were designed to the least degenerate regions of both amino- and carboxy-terminal amino acid sequence. To decrease oligonucleotide redundancy, pools of oligonucleotides were synthesised and inosines substituted in positions of high ambiguity. Two oligonucleotides were designed to the N-terminal region, designated pWAP N1, 5′ TGYGTIAAYGARAGYTGYCC 3′ and pWAP N2, 5′ TGYGTIAAYGAYGARTCITGYCC 3′. A complementary oligonucleotide was designed to the carboxy-terminal amino acid sequence, pWAP C2, 5′ AARCARCAYTTYTTRTTICC 3′. First strand cDNA synthesis was performed with reagents from a Superscript II kit (Gibco-BRL) as per manufacturer's instructions, using 5 μg total RNA from lactating porcine mammary gland. PCR was performed using 10–50% of the first strand reaction as a template with the oligonucleotide combination of pWAP N1/N2 and pWAP C2. PCR cycles consisted of an initial denaturation step at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 10 min. PCR products were analysed by electrophoresis through a 2% agarose gel. The PCR products were extracted from the gel and cloned into the di-deoxythymidine tailed vector pGEM-T (Promega, Madison, WI, USA). The nucleotide sequence of cloned PCR products was determined by cycle sequencing in both directions using T7 and Sp6 dye-primers (ABI) and subsequent analysis on an ABI automated DNA sequencer.

RNA isolation and Northern hybridisation

RNA was extracted from lactating porcine mammary gland, cultured explants and liver tissue

using Trizol reagent (BRL, Bethesda, MD, USA) according to the manufacturer's instructions. The total RNA (5 µg) was electrophoresed through a 1·4% formaldehyde agarose gel and transferred to Zeta Probe GT (Bio-Rad Labs, Richmond, CA, USA) nylon membrane. Membranes were hybridised in 10 ml hybridisation buffer (0·5 M $NaHPO_4$, 7% SDS, 1% BSA, 1 mM EDTA) with random primed α ^{32}P-labelled WAP PCR DNA and cDNAs for porcine β-casein and α-lactalbumin for 16 h at 65 °C. Membranes were washed twice with 0·1 × SSC/0·1% SDS at 60 °C and exposed to Kodak X-OMAT film. Both β-casein and α-lactalbumin cDNAs were isolated in our laboratory using reverse transcriptase (RT)–PCR with RNA from porcine lactating mammary gland and primers designed to published cDNA sequences (Alexander & Beattie 1992*b*, Das Gupta *et al.* 1992)

Mammary gland explant culture

Mammary tissue was obtained by biopsy (King *et al.* 1996) from six Landrace × Large White gilts at day 90 of pregnancy. Approximately 500 mg mammary tissue were pooled from two glands for each sow and transferred immediately to hormone-free medium 199 (M199) at room temperature. The tissue from each animal was cut into explants (approximately 1 mg each), placed onto siliconised lens paper, and cultured in M199 supplemented with various combinations of porcine insulin (I, 1 µg/ml), cortisol (F, 0·05 µg/ml) and porcine prolactin (P, 0·1 µg/ml) for 3 days under 5% CO_2 at 37 °C. The media were changed daily. Tissue (100 mg) from at least three dishes was pooled for each treatment and explants from each animal analysed separately. Tissue was frozen on dry ice prior to culture for the preparation of RNA and for histological examination. Prior to sectioning, tissue was fixed in 10% buffered formalin and set in paraffin blocks. Sections were stained with haematoxylin and eosin to confirm the presence of mammary epithelial tissue.

SDS PAGE analysis of milk proteins

Whole milk samples were collected from sows at intervals from farrowing to weaning (day 21) (Attwood & Hartmann 1992). The whey proteins were isolated as described above and subjected to SDS polyacrylamide gel electrophoresis (Laemmli 1970) in 20% polyacrylamide gels. Proteins were stained with Coomassie blue G250 and destained in 40% methanol/10% acetic acid.

RESULTS

Isolation, cloning and sequence analysis of WAP

Examination of the secretory pattern of WAP by SDS PAGE of whey collected from farrowing to day 21 of lactation showed that WAP is secreted at a consistent level throughout lactation (data not shown). The whey component of milk collected from sows at day 21 of lactation was fractionated on HPLC and separated as five major protein peaks (Fig. 1). SDS PAGE analysis indicated the presence of a 17·5 kDa protein in peak 3 (Fig. 1 inset), eluting at 37·3% acetonitrile. Amino acid sequencing of the protein component of this peak revealed significant homology to the amino- and carboxy-terminal regions of camel WAP.

To complete the region of unknown sequence, degenerate oligonucleotides were designed to the sequenced amino- and carboxy-terminal ends and used to generate a PCR product of the expected size of 162 bp. The PCR product was cloned into a dideoxythymidine tailed vector and the nucleotide sequence of several clones determined in both directions to eliminate any sequencing errors or PCR proof reading errors. The deduced amino acid sequence of the WAP clone together with the determined amino acid sequence indicates a mature protein of 111 amino acids (Fig. 2) with a molecular mass of 11·7 kDa.

A comparison of the amino acid sequence of porcine WAP with those from other species shows the two-domain arrangement, each consisting of eight cysteine residues, characteristic of four-disulphide core proteins (Fig. 3a). Within each domain there is a conserved disulphide core comprising six equally spaced cysteine residues. The remaining two cysteines which complete the folding unit occur at various positions with respect to the conserved disulphide cores. Porcine WAP shows similarity at the amino acid level of 75, 50, 40 and 35% to WAPs from camel, rabbit, rat and mouse respectively. A highly conserved WAP motif, with consensus sequence KAGRCPW, is present in all WAPs at the beginning of the second domain. Alignment of the amino acid sequence of WAPs with proteins from the Kunitz group of protease inhibitors indicates these proteins also exhibit a WAP-like cysteine rich domain and partially conserved motif (Fig. 3b).

Hormone-dependent expression of the WAP gene

Northern analysis of RNA from lactating mammary tissue showed that the porcine WAP cDNA
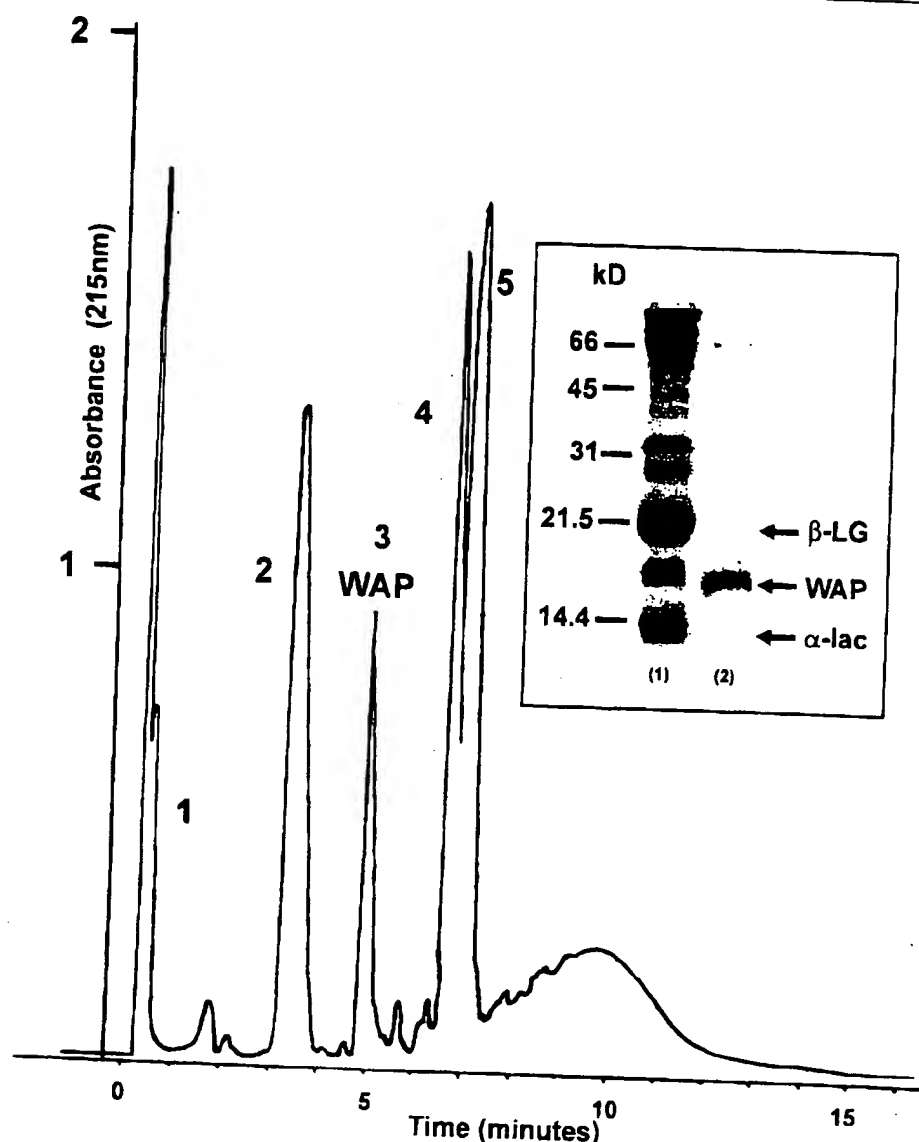
FIGURE 1.   Isolation of WAP from porcine whey by HPLC. Porcine whey (50 µl) was acidified and fractionated by HPLC using a POROS R2/H reverse phase column and a 15–60% gradient of acetonitrile in 0·1% TFA at a flow rate of 4 ml/min. The absorbance of the protein peaks was measured at 215 nm on a full scale of 0 to 2·0. The major peaks of protein are numbered from 1 to 5. The inset shows SDS polyacrylamide gel (20%) electrophoresis of total whey (lane 1) and peak 3 (lane 2) which eluted at 37·3% acetonitrile. The position of WAP and the major whey proteins β-lactoglobulin (β-LG) and α-lactalbumin (α-lac) are indicated by arrows. The positions of the molecular mass markers are indicated at the side by their molecular masses in kDa.

hybridised to a single transcript of 600 bp (Fig. 4). No transcript was detected when using RNA isolated from liver. The hormonal control of WAP gene expression was examined in mammary explants prepared from tissue biopsies taken from sows at 90 days of pregnancy. Examination of sections prepared from mammary tissue prior to culture and stained with haematoxylin and eosin revealed the presence of alveoli with cuboidal

epithelial cells and the absence of secretory activity (data not shown). Northern analysis of the RNA extracted from the tissue prior to culture indicated that WAP gene expression was barely detectable and there was no increment in explants cultured for 3 days in media containing I and F (Fig. 4). However, a significant increase in the accumulation of WAP mRNA was observed in explants cultured in I, F and P. The level of WAP mRNA in explants

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L A P A L N L P G L A T C P E L S S S D P C V I S  C V N D E S C P

    Q       G       T       K       C       C       A       R       S       P       C
C A A G G C A C C A A G T G C T G C G C C A G G A G C C C C T G C

    S       R       S       C       T       V       P       L       L       V       P
A G C C G A T C C T G C A C G G T C C C C C T C C T G G T G C C T

    V       P       K       A       G       R       C       P       W       V       P
G T C C C C A A G G C C G G C C G C T G C C C C T G G G T G C C G

    A       P       L       A       P       E       L       C       L       E       K
G C C C C G C T G G C C C C C T G A G C T C T G C T T G G A G A A A

    N       E       C       S       R       D       D       Q       C       R       G N K
A A T G A G T G C T C C A G G G A C G A C C A G T G T A G G

 K C C F  S S C A M R C L D P D T E A P L Q
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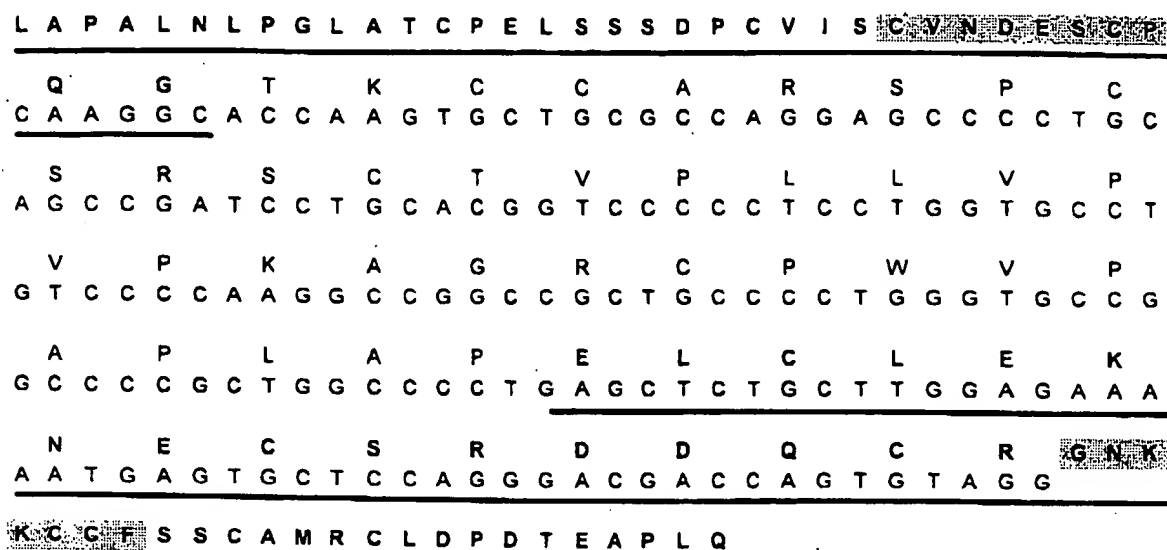
FIGURE 2.   The amino acid and nucleotide sequence of porcine WAP. The amino acid sequence of the amino- and carboxy-terminal regions of pWAP determined by peptide sequencing are underlined. The shaded regions denote the 5' and 3' amino acid sequence from which the degenerate PCR primers were designed. The nucleotide sequence and deduced amino acid sequence of the 162 bp PCR product are shown.

was similar when prolactin was included in media at a concentration of either 0·1 or 1 μg/ml (data not shown). The induction of β-casein and α-lactalbumin gene expression in mammary explants cultured under the same conditions also required the presence of I, F and P in the media (Fig. 4).

DISCUSSION

WAP has been identified as the major whey protein in the milk of the mouse (Hennighausen *et al.* 1982), rat (Campbell *et al.* 1984), rabbit (Devinoy *et al.* 1988) and camel (Beg *et al.* 1986). In contrast, β-lactoglobulin has been identified as the major whey protein secreted by most livestock species (Hambling *et al.* 1992), and to date there has been no evidence for the presence of WAP in milk of these species. The camel may be considered a livestock species in some countries but there is no convincing evidence showing that β-lactoglobulin is secreted in the milk of this species. This paper provides the first report that WAP is a major whey protein in the milk of the pig, indicating that the secretion of this protein may be more widespread than generally accepted.

The WAP family of proteins and the Kunitz-type protease inhibitors show limited amino acid sequence similarity across the entire molecule, but significant conservation of cysteine residues within

a two-domain arrangement, consistent with the hypothesis that the gene for these proteins arose by intragenic duplication during evolution (Hennighausen *et al.* 1982). A conserved motif of seven amino acids, KAGRCPW, is located at the beginning of the second domain and shows significant identity at the amino acid level in all WAPs with the exception of the rabbit (Thepot *et al.* 1990) and camel (Beg *et al.* 1986) which differ by a single amino acid substitution. This motif is also highly conserved in the Kunitz-type protease inhibitors and is most likely implicated in the biological functions of these proteins. Porcine WAP conforms to the characteristic two-domain structure showing conservation of cysteine residues and limited sequence similarity with all the species with the exception of camel WAP. It is interesting to speculate that this similarity may relate to their common evolutionary origin (Novacek 1992). However, cows also originated from the same order artiodactyla (even toed ungulates) and although they secrete a protein with characteristics of a Kunitz-type protease inhibitor in the colostrum (Cechova *et al.* 1971), there is, as yet, no evidence of WAP secretion in the milk.
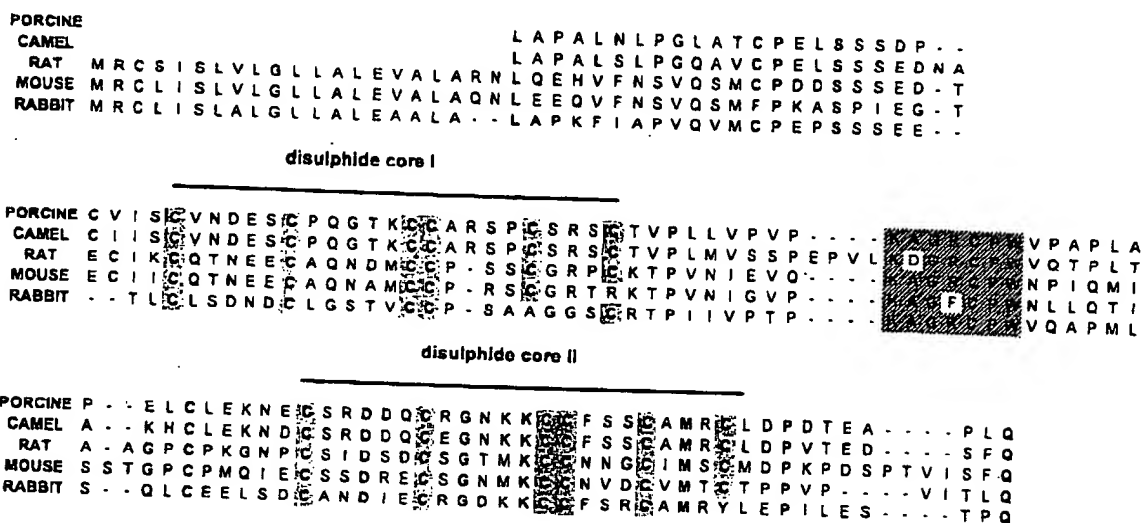
The majority of the protease inhibitors that fall into the WAP class contain only one of these cysteine rich domains which tends to align with the more conserved disulphide core II of WAP from all species. The recent finding of porcine homologues for human elafin which contain a single WAP

domain, showed that there are a variety of polymorphisms associated with these protease inhibitors (Tamechika et al. 1996). However, based on similarity to the WAP cysteine rich disulphide core, these proteins were incorrectly named pig WAPs since they exhibit very little sequence similarity to porcine WAP.
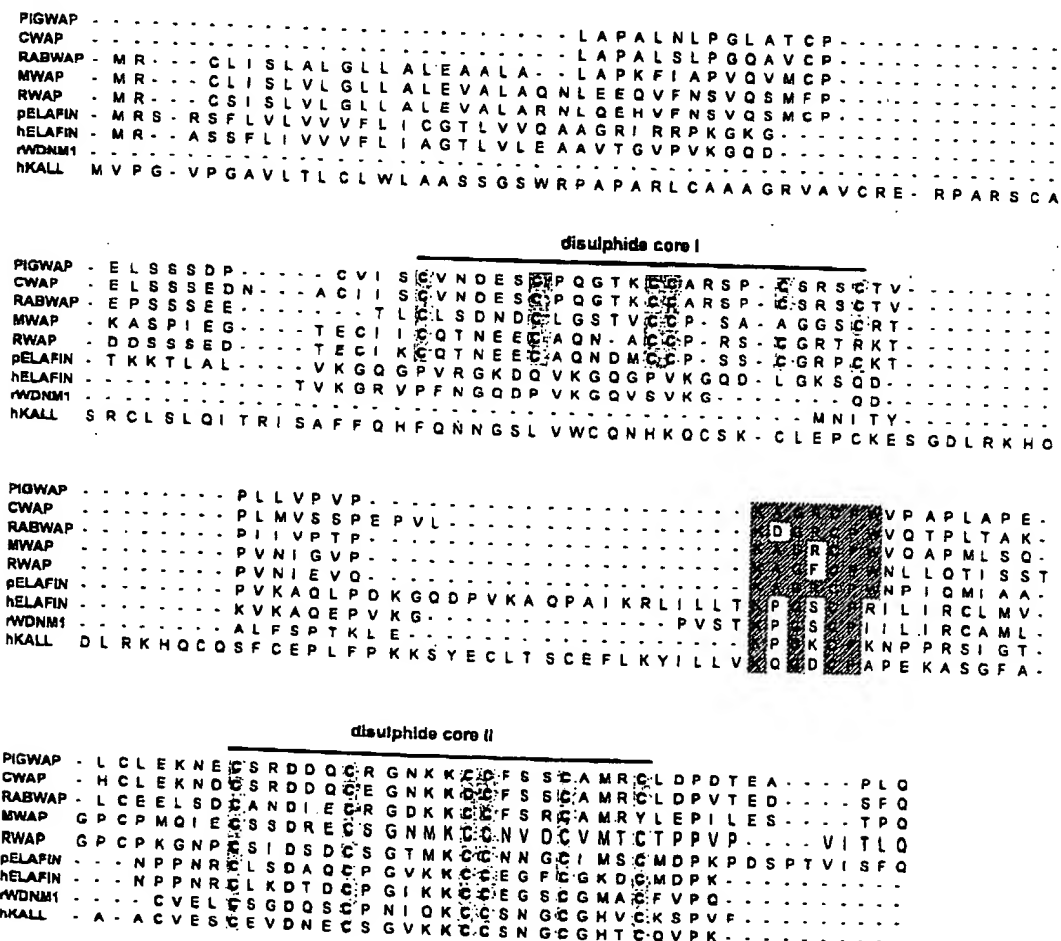
Experiments designed to examine the hormonal control of the WAP gene in mammary explants from mid-pregnant mice and rats have shown a
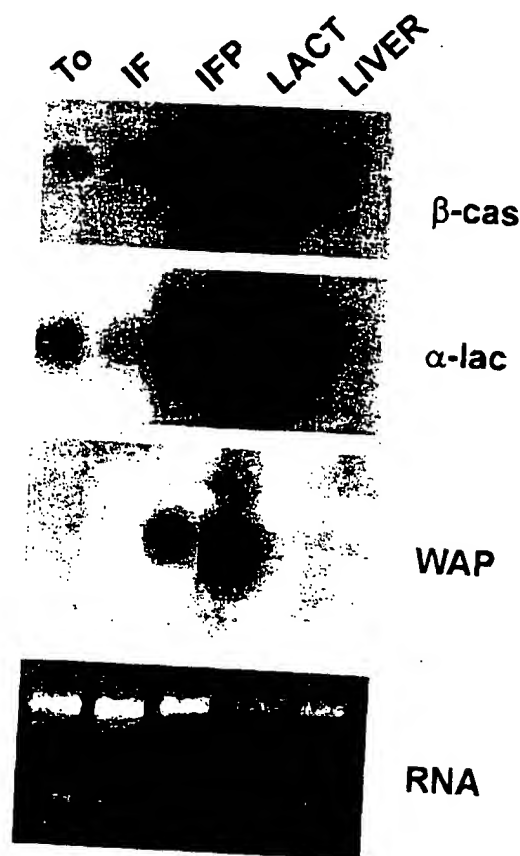
(a)

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PORCINE
CAMEL                                    L A P A L N L P G L A T C P E L S S S D P . .
RAT     M R C S I S L V L G L L A L E V A L A R N L Q E H V F N S V Q S M C P D D S S S E D N A
MOUSE   M R C L I S L V L G L L A L E V A L A Q N L E E Q V F N S V Q S M F P K A S P I E G . T
RABBIT  M R C L I S L A L G L L A L E A A L A . . L A P K F I A P V Q V M C P E P S S S E E . .

                          disulphide core I
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PORCINE C V I S C V N D E S C P Q G T K C C A R S P C S R S C T V P L L V P V P . . .        V P A P L A
CAMEL   C I I S C V N D E S C P Q G T K C C A R S P C S R S C T V P L M V S S P E P V L      V Q T P L T
RAT     E C I K C Q T N E E C A Q N D M C C P . S S C G R P C K T P V N I E V Q . . . D      N P I Q M I
MOUSE   E C I I C Q T N E E C A Q N A M C C P . R S C G R T R K T P V N I G V P . . .        N L L Q T I
RABBIT  . . T L C L S D N D C L G S T V C C P . S A A G G S C R T P I I V P T P . . . F      V Q A P M L

                          disulphide core II
                     _____

PORCINE P . . E L C L E K N E C S R D D Q C R G N K K C C F S S C A M R C L D P D T E A . . . . P L Q
CAMEL   A . . K H C L E K N D C S R D D Q C E G N K K C C F S S C A M R C L D P V T E D . . . . S F Q
RAT     A . A G P C P K G N P C S I D S D C S G T M K C C N N G C I M S C M D P K P D S P T V I S F Q
MOUSE   S S T G P C P M Q I E C S S D R E C S G N M K C C N V D C V M T C T P P V P . . . . V I T L Q
RABBIT  S . . Q L C E E L S D C A N D I E C R G D K K C C F S R C A M R Y L E P I L E S . . . . T P Q
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(b)

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PIGWAP  . . . . . . . . . . . . . . . . . . . . . . . . . . . . L A P A L N L P G L A T C P . . . . . . . .
CWAP    . . . . . . . . . . . . . . . . . . . . . . . . . . . . L A P A L S L P G Q A V C P . . . . . . . .
RABWAP  . M R . . . C L I S L A L G L L A L E A A L A . . L A P K F I A P V Q V M C P . . . . . . . .
MWAP    . M R . . . C L I S L V L G L L A L E V A L A Q N L E E Q V F N S V Q S M C P . . . . . . . .
RWAP    . M R . . . C S I S L V L G L L A L E V A L A R N L Q E H V F N S V Q S M F P . . . . . . . .
pELAFIN . M R S . R S F L V L V V V F L I C G T L V V Q A A G R I R R P K G K G . . . . . . . .
rWDNM1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
hKALL   M V P G . V P G A V L T L C L W L A A S S G S W R P A P A R L C A A A G R V A V C R E . R P A R S C A

                                          disulphide core I
                                      _____

PIGWAP  . E L S S S D P . . . . . C V I S C V N D E S C P Q G T K C C A R S P . C S R S C T V . . . . . . . .
CWAP    . E L S S S E D N . . . . . A C I I S C V N D E S C P Q G T K C C A R S P . C S R S C T V . . . . . . . .
RABWAP  . E P S S S E E . . . . . . . . T L C L S D N D C L G S T V C C P . R S . C S R S C T V . . . . . . . .
MWAP    . K A S P I E G . . . . . T E C I I C Q T N E E C A Q N . A C C P . R S . A G G S C R T . . . . . . . .
RWAP    . D D S S S E D . . . . . T E C I K C Q T N E E C A Q N D M C C P . S S . C G R T R K T . . . . . . . .
pELAFIN T K K T L A L . . . . . V K G Q G P V R G K D Q V K G Q G P V K G Q D . L G K S Q D . . . . . . . .
hELAFIN . . . . . . . . . . . . T V K G R V P F N G Q D P V K G Q V S V K G . . . . . . Q D . . . . . . . .
rWDNM1  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . Q D . . . . . . . .
hKALL   S R C L S L Q I T R I S A F F Q H F Q N N G S L V W C Q N H K Q C S K . C L E P C K E S G D L R K H C

PIGWAP  . . . . . . . . P L L V P V P . . . . . . . . . . . . . . . . . . . . . . V P A P L A P E .
CWAP    . . . . . . . . P L M V S S P E P V L . . . . . . . . . . . . . . . . . . V Q T P L T A K .
RABWAP  . . . . . . . . P I I V P T P . . . . . . . . . . . . . . . . . . R . . . V Q T P L T A K .
MWAP    . . . . . . . . P V N I G V P . . . . . . . . . . . . . . . . . . F . . . V Q A P M L S Q .
RWAP    . . . . . . . . P V N I E V Q . . . . . . . . . . . . . . . . . . . . . . N L L Q T I S S T
pELAFIN . . . . . . . . P V K A Q L P D K G Q D P V K A Q P A I K R L I L L T P . S . . N P I Q M I A A .
hELAFIN . . . . . . . . K V K A Q E P V K G . . . . . . . . . . . . . . . . P . S . . R I L I R C L M V .
rWDNM1  . . . . . . . . A L F S P T K L E . . . . . . . . . . . . . . P V S T P . K . . I I L I R C A M L .
hKALL   D L R K H Q C Q S F C E P L F P K K S Y E C L T S C E F L K Y I L L V Q . D . D . A P E K A S G F A .

                                          disulphide core II
                                      _____

PIGWAP  . L C L E K N E C S R D D Q C R G N K K C C F S S C A M R C L D P D T E A . . . . P L Q
CWAP    . H C L E K N D C S R D D Q C E G N K K C C F S S C A M R C L D P V T E D . . . . S F Q
RABWAP  . L C E E L S D C A N D I E C R G D K K C C F S R C A M R Y L E P I L E S . . . . T P Q
MWAP    G P C P M Q I E C S S D R E C S G N M K C C N V D C V M T C T P P V P . . . . V I T L Q
RWAP    G P C P K G N P C S I D S D C S G T M K C C N V D C V M T C T P P V P . . . . V I T L Q
pELAFIN . . . N P P N R C L S D A Q C P G V K K C C E G F C G K D C M D P K P D S P T V I S F Q
hELAFIN . . . N P P N R C L K D T D C P G I K K C C E G S C G M A C F V P Q . . . . . . . . .
rWDNM1  . . . . C V E L S G D Q S C P N I Q K C C S N G C G H V C K S P V F . . . . . . . . .
hKALL   . A . A C V E S C E V D N E C S G V K K C C S N G C G H T C Q V P K . . . . . . . . .
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FIGURE 4. Hormone-dependent expression of porcine milk protein genes. Mammary tissue was obtained by biopsy from Landrace × Large White gilts at day 90 of pregnancy. Mammary tissue was pooled from two glands for each sow and explants cultured in M199 with the indicated combinations of porcine insulin (I, 1 µg/ml), cortisol (F, 0·05 µg/ml) and porcine prolactin (P, 0·1 µg/ml) for 3 days under 5% CO_2 at 37 °C. Results for tissue collected at the time of culture (To) is shown. RNA was extracted from the mammary gland of a sow at day 21 of lactation (LACT) and the liver (LIVER) of the same animal. Total RNA (5 µg) was analysed by Northern analysis using cDNA probes for porcine whey acidic protein (WAP), α-lactalbumin (α-lac) and β-casein (β-cas).

shown that the WAP gene is induced in the presence of prolactin alone but that the level of induction is significantly enhanced if cortisol and insulin are also present in the culture media (Puissant & Houdebine 1991). More recently the transgenic pig has been used as a model to study the expression of a 7·2 kb mouse WAP transgene based on the assumption that there is no endogenous porcine WAP gene expression. Shamay et al. (1991) and Wall et al. (1991) generated lines of transgenic pigs expressing the mouse WAP gene and analysed the milk proteins secreted by the transgenic sows by Western blot with anti-mouse WAP antibodies. Interestingly, a protein of 14 kDa which corresponds to the mouse WAP protein, and an additional protein of approximately 20 kDa were identified. It is conceivable that this larger protein could be porcine WAP which may share epitopes with mouse WAP (Shamay et al. 1991) that are recognised by the polyclonal antiserum. However, hybridisation of lactating mammary gland RNA from non-transgenic sows with mouse WAP cDNA did not reveal the presence of a transcript (Shamay et al. 1991, 1992a,b) and may be attributed to stringent washing and low homology between genes from these two species.

Studies directed to understanding the endocrine control of mouse WAP transgene expression in mammary explants from these transgenic pigs showed considerable variability in the requirement for insulin, cortisol and prolactin (Shamay et al. 1992b), suggesting that the 7·2 kb WAP gene clone did not include all the necessary regulatory elements (Shamay et al. 1992b). However, expression of the porcine β-casein gene in the same explants required all three hormones in the culture media (Shamay et al. 1992b) and a similar requirement was reported for the expression of α-lactalbumin (Shamay et al. 1992a). In addition, a recent report (Dodd et al. 1994) has shown that the production of α-lactalbumin is induced in mammary explants from sows at 90 days of gestation only when insulin, cortisol and prolactin are present in media. The current study confirms both these observations for the hormonal control of the β-casein and α-lactalbumin gene, and in addition, clearly

requirement for insulin, cortisol and prolactin for induction of the gene (Hobbs et al. 1982, Pittius et al. 1988). Similar experiments in the rabbit have

FIGURE 3. Comparison of the amino acid sequence of WAP from the pig (PIGWAP), camel (CWAP), rat (RWAP), mouse (MWAP) and rabbit (RABWAP) and a range of protease inhibitors. (a) The amino acid sequence of the two conserved disulphide cores characteristic of the four-disulphide core proteins of WAP (shaded) and the KAGRCPW motif (hatched area) are shown. The additional two cysteine residues which constitute the four-disulphide core folding domain and other conserved amino acids are shown in bold. (b) An alignment of amino acid sequence of all WAPs with various protease inhibitors which fall into the four-disulphide group of proteins; rat WDNM1 (rWDNM1) (Dear & Kefford 1991), human Kallmann syndrome (hKALL) (Legouis et al. 1991), human elafin (hELAFIN) (Wideow et al. 1990) and porcine elafin (pELAFIN) (Tamechika et al. 1996) is shown. The cysteine rich disulphide cores and the conserved motif are identified as for Fig. 3a.

demonstrates that the porcine WAP gene is also controlled by the combination of insulin, cortisol and prolactin.

The function of WAP remains to be determined although some evidence suggests that it may play an important role in mammary gland development and tissue remodelling (McKnight *et al.* 1991). For example, the overexpression of a WAP transgene in both mice and pigs during early pregnancy adversely affected mammary epithelial cell differentiation and ultimately, function of the mammary gland (Burdon *et al.* 1991, Shamay *et al.* 1992a). A similar putative developmental function has been assigned to another member of the four-disulphide core protein family which suppressed growth of a tumour cell line (Dear & Kefford 1991) suggesting it too may act as a negative regulator. The significance of the similarity of WAP with the four-disulphide core protease inhibitor family remains to be elucidated, particularly as WAP appears unable to elicit any general protease inhibitor activity (Burdon *et al.* 1991). However, this does not preclude the possibility of inhibitory action which is directed against very specific proteases with a subsequent effect on mammary gland development or development of the young.

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REFERENCES

Alexander LJ & Beattie CW 1992a The sequence of porcine alpha S1-casein cDNA: evidence for protein variants generated by altered RNA splicing. *Animal Genetics* 23 283–288.

Alexander LJ & Beattie CW 1992b The sequence of porcine β-casein cDNA. *Animal Genetics* 23 369–371.

Alexander LJ & Beattie CW 1992c Sequence of porcine β-lactoglobulin cDNA. *Animal Genetics* 23 263–265.

Alexander LJ, Das Gupta NA & Beattie CW 1992 The sequence of porcine alpha S2-casein cDNA. *Animal Genetics* 23 356–367.

Attwood CS & Hartmann PE 1992 Collection of fore and hind milk from the sow and changes in milk composition during suckling. *Journal of Dairy Research* 59 287–298.

Beg OU, Von Bahr-Lindstrom H, Zaidi ZH & Jornvall H 1986 A camel milk whey protein rich in half-cysteine: primary structure, assessment of variations, internal repeat patterns, and relationships with neurophysin and other active polypeptides. *European Journal of Biochemistry* 159 195–201.

Burdon T, Sankaran L, Wall RJ, Spencer M & Hennighausen L 1991 Expression of a whey acidic protein transgene during mammary development: evidence for different mechanisms of regulation during pregnancy and lactation. *Journal of Biological Chemistry* 266 6909–6914.

Campbell SM, Rosen JM, Hennighausen LG, Strech-Jurk U & Sippel AE 1984 Comparison of the whey acidic protein genes of the rat and mouse. *Nucleic Acids Research* 12 8685–8697.

Cechova D, Jonaka V & Sorm F 1971 Primary structure of trypsin inhibitor from cow colostrum (component B2). *Collection of Czechoslovakian Chemistry Communications* 36 3342–3357.

Das Gupta NA, Alexander LJ & Beattie CW 1992 The sequence of a porcine cDNA encoding alpha-lactalbumin. *Gene* 110 265–266.

Dear TN & Kefford F 1991 The WDNM1 gene product is a novel member of the 'four-disulphide core' family of proteins. *Biochemical and Biophysical Research Communications* 176 247–254.

Devinoy E, Hubert C, Jolivet G, Thepot D, Clergue N, Desaleux M, Dion M, Servely JL, & Houdebine LM 1988 Recent data on the structure of rabbit milk protein genes and on the mechanism of the hormonal control of their expression. *Reproduction, Nutrition and Development* 28 1145–1164.

Dodd SC, Forsyth IA, Buttle HL, Gurr MI & Dils RR 1994 Hormonal induction of α-lactalbumin and β-lactoglobulin in cultured mammary explants from pregnant pigs. *Journal of Dairy Research* 61 35–45.

Grabowski H, Le Bars D, Chene N, Attal J, Malienou-Ngassa R, Puissant C & Houdebine LM 1991 Rabbit whey acidic protein concentration in milk, serum, mammary gland extract and culture medium. *Journal of Dairy Science* 74 4142–4150.

Groenen M-AM & van der Poel JJ 1994 Regulation of milk protein genes: a review. *Livestock Production Science* 38 61–78.

Hambling SG, McAlpine AS & Sawyer L 1992 β-lactoglobulin. In *Advanced Dairy Chemistry 1: Proteins*, pp 141–190. Ed PF Fox. Oxford: Elsevier Science Publishers Ltd.

Hennighausen LG & Sippel AE 1982 Mouse whey acidic protein is a novel member of the family of 'four-disulphide core' proteins. *Nucleic Acids Research* 10 2677–2684.

Hennighausen LG, Sippel AE, Hobbs AA & Rosen JM 1982 Comparative sequence analysis of the mRNAs coding for mouse and rat whey acidic protein. *Nucleic Acids Research* 10 3733–3744.

Hobbs AA, Richards DA, Kessler DJ & Rosen JM 1982 Complex hormonal regulation of rat casein gene expression. *Journal of Biological Chemistry* 257 3598–3605.

King RH, Pettigrew JE, McNamara JP, McMurty JP, Henderson TL, Hathaway MR & Sower AF 1996 The effect of endogenous prolactin on lactation performance of first-litter sows given protein-deficient diets during the first pregnancy. *Animal Production Science* 41 37–50.

Krozowski Z 1989 Generation of polyclonal antibodies against purified rat whey acidic proteins and the synthesis of a tracer fusion protein suitable for use in radioimmunoassay. *Molecular and Cellular Endocrinology* 63 15–22.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 680–685.

Legouis R, Hardelin JP, Levilliers J, Claverie JM, Compain S, Wunderle V, Millasseau P, Lepaslier D, Cohen D, Caterina D, Bougueleret L, Delmarre-Van de Waal H, Lutfalla G, Weissenbach J & Petit C 1991 The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell* 67 423–435.

Levine WB, Alexander LJ, Hoganson GE & Beattie CW 1992 Cloning and sequence of the porcine kappa-casein cDNA. *Animal Genetics* 23 361–363.

McKnight RA, Burdon T, Pursel VG, Shamay A, Wall RJ & Hennighausen L 1991 The whey acidic protein. In *Genes,*

Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer, pp 399–412. Eds RB Dickson & ME Lippman. Boston, USA: Kluwer Academic Publishers.

Nicholas KR, Wilde CJ, Bird PH, Hendry KAK, Tregenza K & Warner B 1995 Asynchronous concurrent secretion of milk proteins in the tammar wallaby (*Macropus eugenii*). In *Intercellular Signalling in the Mammary Gland*, pp 153–170. Eds CJ Wilde, M Peaker & C Knight. New York: Plenum Publishing Corporation.

Novacek MJ 1992 Mammalian phylogeny: shaking the tree *Nature* 356 121–125.

Pittius CW, Sankaran L, Topper YJ & Hennighausen L 1988 Comparison of the regulation of the whey acidic protein gene with that of a hybrid gene containing the whey acidic protein gene promoter in transgenic mice. *Molecular Endocrinology* 2 1027–1032.

Puissant C & Houdebine LM 1991 Cortisol induces rapid accumulation of whey acidic protein mRNA but not of αs1-casein and β-casein mRNA in rabbit mammary explants. *Cell Biology International Reports* 15 121–129.

Shamay A, Solinas S, Pursel VG, McKnight RA, Alexander L, Beattie C, Hennighausen L & Wall RJ 1991 Production of the mouse whey acidic protein in transgenic pigs during lactation. *Journal of Animal Science* 69 4552–4562.

Shamay A, Pursel VG, Wilkinson E, Wall RJ & Hennighausen L 1992a Expression of the whey acidic protein in transgenic pigs impairs mammary development. *Transgenic Research* 1 124–132.

Shamay A, Pursel VG, Wall RJ & Hennighausen L 1992b Induction of lactogenesis in transgenic virgin pigs: evidence for gene and integration site-specific hormonal regulation. *Molecular Endocrinology* 6 191–197.

Shaw DC, Messer M, Scrivener AM, Nicholas KR & Griffiths M 1993 Isolation, partial characterisation and amino acid sequence of α-lactalbumin from platypus (*Ornithorhynchus*

anatinus) milk. *Biochimica et Biophysica Acta* 1161 177–186.

Tamechika I, Itakura M, Suta Y, Furukawa M, Kato A, Tachibana S & Hiroses S 1996 Accelerated evolution in inhibitor domains of porcine elafin family members. *Journal of Biological Chemistry* 271 7012–7018.

Thepot D, Devinoy E, Fontaine ML, Hubert C & Houdebine LM 1990 Complete sequence of the rabbit whey acidic protein gene. *Nucleic Acids Research* 18 3641.

Velander WH, Johnson JL, Page RL, Russell CG, Subramanian A, Wilkins TD, Gwazadauskas FC, Pittius C & Drohan WN 1992 High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proceedings of the National Academy of Science of the USA* 89 12003–12007.

Wall RJ, Pursel VG, Shamay A, McKnight RA, Pittius CW & Hennighausen L 1991 High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. *Proceedings of the National Academy of Sciences of the USA* 88 1696–1700.

Wideow O, Schroder J-M, Gregory H, Young JA & Christophers E 1990 Elafin: an elastase-specific inhibitor of human skin. *Journal of Biological Chemistry* 265 14791–14795.

NOTE ADDED IN PROOF

The full length porcine WAP cDNA has subsequently been cloned and sequenced. It is available from the EMBL database under accession number AJ000221.